

Continuation of U.S.S.N. 10/006,915
Filed: February 4, 2004
PRELIMINARY AMENDMENT
Express Mail Label No.: EL 717 745 679 US
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In the specification

Please replace the paragraph on page 1, lines 5-8. with the following paragraph:

This application is a continuation of U.S. Serial No. 10/006,915 filed November 9, 2001,
which is a continuation of U.S. Serial No. 09/156,809 filed September 18, 1998 (now U.S. Patent
No. 6,316,262), which claims priority to U.S. Serial No. 60/059,373 filed September 19, 1997,
entitled Biological Systems for the Manufacture of Polyhydroxyalkanoate Polymers containing
4-Hydroxyacids by Gjalt W. Huisman, Frank A. Skraly, David P. Martin, and Oliver P. Peoples.

Please replace the paragraph on page 6, lines 16-21, with the following paragraph:

Figure 1A is the alignment of the *C. kluyveri* OrfZ sequence with the N-terminal sequence and internal sequences of 4-hydroxybutyryl CoA transferase (4HBCT) from *C. aminobutyricum* (SEQ ID Nos 1 and 2. Identical residues are indicated, similar residues are indicated by *. Figure 1B is Figure 1B and Figure 1C are the nucleotide sequence of the *orfZ* gene from *C. kluyveri kluyveri* (SEQ ID NO:3). Figure 1C 1D is the amino acid sequence of the *orfZ* gene from *C. kluyveri kluyveri* (SEQ ID NO:1).

Please replace the paragraph on page 7, lines 9-12, with the following paragraph:

Figure 6 is Figure 6 and Figure 6A are a schematic of the construction of plasmids for integration of 3-ketoacyl-CoA thiolase (*phbA*) and acetoacetyl-CoA reductase (*phbB*) genes from *Z. ramigera* into the chromosome of *E. coli* and other Gram-negative bacteria.

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Please replace the paragraph bridging pages 22 and 23 with the following paragraph:

Plasmid pMUXC₅cat contains the *phbC* gene from *Z. ramigera* on a transposable element for integration of this gene on the chromosome of a recipient strain (Figure 5). Strong translational sequences were obtained from pKPS4 which encodes PHA synthase encoding *phaC1* from *P. oleovorans* in the pTrc vector (Pharmacia). In this construct, *phaC1* is preceded by a strong ribosome binding site: AGGAGGTTTT(-ATG) (SEQ ID NO:4). The *phaC1* gene, including the upstream sequences, was cloned as a blunt ended *EcoRI-HindIII* fragment in the *SmaI* site of pUC18Sfi to give pMSXC₃. A blunt ended *cat* gene cassette was subsequently cloned in the blunt-ended *Sse8387II* site, resulting in pMSXC₃cat. At this point, all of the *phaC1* coding region except the 5' 27 base pairs were removed as a *PstI-BamHI* fragment and replaced by the corresponding fragment from the *phbC* gene from *Z. ramigera*. The resulting plasmid, pMSXC₅cat, encodes a hybrid PHB synthase enzyme with the 9 amino terminal residues derived from the *P. oleovorans* PHA synthase and the remainder from *Z. ramigera*. The C₅cat cassette was then excised as an *AvrII* fragment and cloned in the corresponding sites of pUTHg, thereby deleting the mercury resistance marker from this vector. The resulting plasmid, pMUXC₅cat, contains a C₅cat mini-transposon in which *phbC* is not preceded by a promoter sequence. Expression of the cassette upon integration is therefore dependent on transcriptional sequences that are provided by the DNA adjacent to the integration site.

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Please replace the paragraphs on page 23, lines 25-29, with the following paragraphs:

3A (5'GGCTCGTATAATGTGTGGAGGGAGAACCGCCGGCTCGGCCGTT)

(SEQ ID NO:5) and

3B (5' CTAGAACGGCGCGAGCCCGGCGTTCTCCCTCCACA CATTATACGA

GCCTGCA) (SEQ ID NO:6).

Please replace the paragraph bridging pages 23 and 24 with the following paragraph:

Next, a fragment containing a consensus *E. coli pho* box and -35 promoter region were inserted into the *PstI* site as a fragment obtained after annealing the oligonucleotides:

2A: (5' TCCCC TGTATAAAGTTGTCAGTGCA) (SEQ ID NO:7) and

2B (5' GTGACAACTTATGACAGGGG ATGCA) (SEQ ID NO:8). Next, the messenger stabilizing sequence including the transcriptional start site from AB₅ was inserted into the *XbaI*-*NdeI* sites as a fragment obtained after annealing the oligonucleotides: 4A (5': CTAGTGCCGG ACCCGGTTCCAAGGCCGGCGCAAGGCTGCCAGAACTGAGGAAGCACA)

(SEQ ID NO:9) and

4B: (5'TATGTGCTTCCTCAGTTCTGGCAGCCTGGCGGCCCTGGAA

CCGGGTCCGGCA) (SEQ ID NO:10). The resulting plasmid is pMSXp₁₂AB₅kan2. The *AvrII* fragment, containing Tp₁₂AB₅kan2 was cloned into pUTHg cut with *AvrII* and used for integration into the genome of MBX379 and MBX245.

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Please replace the paragraphs on page 30, lines 27-30, with the following paragraphs:

4HBD-N: 5' CTCTGAATTCAAGGAGGAAAAAATATGAAGTTAT

TAAAATTGGC (*EcoRI*) (SEQ ID NO:11)

4HBD-C: 5' TTTCTCTGAGCTCGGGATATTAAATGATTGTAGG

(*SacI*) (SEQ ID NO:12).

Please replace the paragraphs on page 31, lines 24-26, with the following paragraphs
(noting that a portion of the sequence in each of lines 24 and 26 was *underlined in the original*):

GH-Up: 5' AACGAATTCAATTCAAGGAGGTTTTATGGATCAGAC

ATATTCTCTGGAGTC (*EcoRI*) (SEQ ID NO:13)

GH-Dn: 5' TTGGGAGCTCTACAGTAAGAAATGCCGTTGG (*SacI*) (SEQ ID NO:14).

Please replace the paragraphs on page 31, lines 30-33, with the following paragraphs:

GB-Up: 5' TAAGAAGCTCAATTCAAGGAGGTTTTATGGATAAGAA

GCAAGTAACGGATTAAAGG (*SacI*) (SEQ ID NO:15)

GB-Dn: 5' TTCCCCGGGTTATCAGGTATGCTGAAGCTGTTCTGT

TGGGC (*XmaI*) (SEQ ID NO:16).

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Please replace the paragraphs on page 32, lines 3-6, with the following paragraphs
(noting that a portion of the sequence in each of lines 3 and 5 was *underlined in the original*):

GT-Up: 5' TCCGGATCCAATTCAGGAGGTTTTATGAACAGCAA

TAAAGAGTTAATGCAG (BamH1) (SEQ ID NO:17)

GT-Dn: 5' GATTCTAGATAGGAGCGGCGCTACTGCTTCGCC (XbaI) (SEQ ID NO:18).